REVIEW

INHIBITORS OF ANGIOGENESIS

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Angiogenesis, the formation of new capillaries, is essential to a number of important physiological events, both normal and pathological. Recently, increased attention has focused on the purification and characterization of inhibitors of this process, because of the potential therapeutic value of angiogenesis inhibitors in controlling such "angiogenic diseases" as proliferative retinopathy, solid tumors, rheumatoid arthritis, and neovascular glaucoma. We review the process of neovascularization and the assays that have been developed to study its inhibition *in vivo* and *in vitro*. We also discuss the properties of different angiogenesis inhibitors and examine the mechanisms by which such inhibitors could potentially intervene in the process of neovascularization.

Angiogenesis or neovascularization, the process of new blood vessel formation, is a very tightly controlled process that rarely occurs under normal conditions, except for instances of wound healing, embryonic development and development of the corpus luteum. Otherwise, vascular turnover is very low. It has been demonstrated that the potential doubling time of capillary endothelium from normal tissues is in the range of 47–20,000 days, as opposed to 2.14–13 days for tumor capillary endothelium[1]. In fact, capillary endothelial cells of most normal tissues are considered to be "quiescent".

However, there exist a growing number of diseases (Table 1) characterized by the pathological growth of new capillaries, which are now considered to be "angiogenic diseases"[2]. These diseases include solid tumors, rheumatoid arthritis, psoriasis and a large number of eye diseases including the proliferative retinopathies, neovascular glaucoma, ocular tumors, e.g. retinoblastoma, as well as a large number of diseases associated with corneal vascularization. For example, some 20 other eye diseases are associated with choroidal neovascularization[3], more than 40 diseases are associated with iris neovascularization[4] and retrolental fibroplasia and uveitis are also considered to be angiogenesis-associated diseases.

What is/are the mechanism(s) by which angiogenesis is controlled? In light of the existence of such a large number of diverse angiogenesis stimulators (for review see ref. 5), it is striking that neovascularization rarely occurs normally. There is an accumulating body of literature which suggests that in addition to growth factors, inhibitors of angiogenesis are key regulators of vascular growth.

The availability of a chemical agent which could prevent the continued spread of vascularization would potentially have broad applicability as a therapy for those diseases in which neovascularization plays a prominent role. For example, in nearly all of the eye diseases mentioned above, once neovascularization occurs, the current methods of treatment are often inadequate to prevent further vascular proliferation. Usually, the first line of treatment is directed to the underlying condition and may involve antibiotics, steroids, etc. As vascularization progresses, cautery or photocoagulation is often utilized. At further stages, this type of therapy is increased in frequency and dosage but has often not been satisfactory. Another clinical arena in which an inhibitor of angiogenesis could play a critical role is the control of solid tumors, since tumor growth is angiogenesis-dependent[6] and tumor angiogenesis has been shown to be permissive for metastasis[7]. It has been suggested that an angiogenesis inhibitor could be administered after excision of a primary tumor to prevent metastatic foci from becoming vascularized or used against primary tumors, in particular against highly vascularized yet inoperable tumors (e.g. brain tumors). Additionally, these inhibitors might be used as an adjunct to chemotherapy or immunotherapy[8].

THE ANGIOGENIC PROCESS AND *IN VIVO* ASSAYS FOR ANGIOGENESIS INHIBITORS

In the early 1970's, a number of *in vivo* angiogenesis assays had been developed and were routinely used. These model systems included the rabbit corneal pocket, the chick chorioallantoic membrane (CAM), the rat dorsal air sac and rabbit ear chamber (for further review see ref. 7). Critical to the use of many of these assays was the development of controlled release polymers capable of releasing large molecules such as angiogenesis stimulators and inhibitors[9]. The two most commonly used *in vivo* assays are the rabbit corneal pocket model and the CAM assay. In the first assay, polymer pellets of ethylene vinyl acetate (EVAc) copolymer are impregnated with test substance[10] and surgically implanted in a pocket in the rabbit

TABLE 1. Angiogenesis dependent diseases.

1.	Angiofibroma
2.	Arteriovenous malformations
3.	Arthritis
4.	Atherosclerotic plaques
5.	Corneal graft neovascularization
6.	Delayed wound healing
7.	Diabetic retinopathy
8.	Granulations—burns
9.	Hemangioma
10.	Hemophilic joints
11.	Hypertrophic scars
12.	Neovascular glaucoma
13.	Nonunion fractures
14.	Osler-Weber Syndrome
15.	Psoriasis
16.	Pyogenic granuloma
17.	Retrolental fibroplasia
18.	Scleroderma
19.	Solid tumors
20.	Trachoma
21.	Vascular adhesions

From ref. 51.

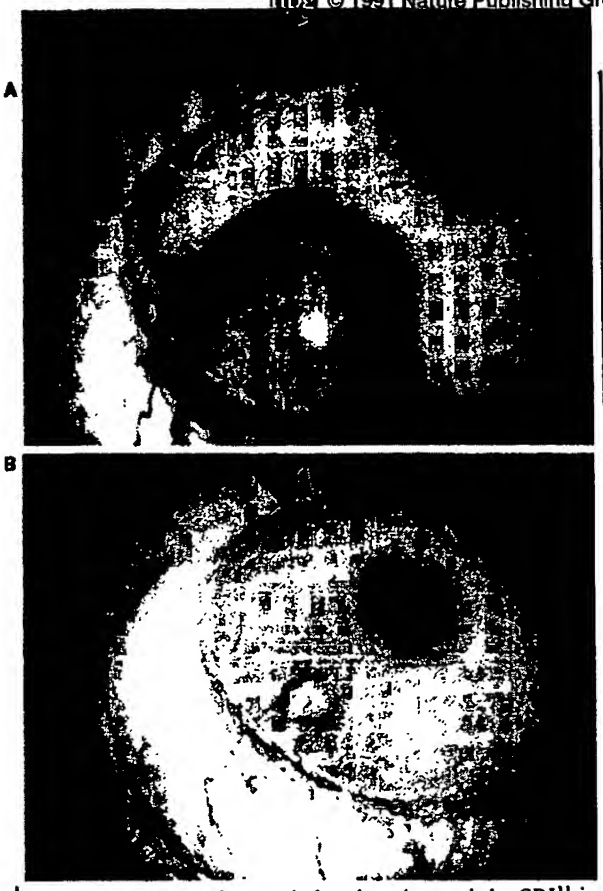

FIGURE 1 Inhibition of tumor-induced angiogenesis by CDI[11] in the rabbit corneal pocket assay. (A) Control cornea implanted with empty EVAc polymer pellet juxtaposed between the limbus of the eye and the V2 carcinoma implant. Capillaries appear as a thick carpet sweeping over polymer and tumor. (B) Test cornea implanted with EVAc polymer pellet impregnated with CDI and juxtaposed between the limbus and the V2 carcinoma implant. Test corneas showed significant inhibition of vessel growth towards the tumor.

cornea approximately 1 mm from the limbus. When this assay system is being used to test for angiogenesis inhibitors, either a piece of V2 carcinoma or some other angiogenic stimulant is implanted distal to the polymer, 2 mm from the limbus. In the opposite eye of each rabbit, control polymer pellets that are empty are implanted next to an angiogenic stimulant in the same way. In these control corneas, capillary blood vessels start growing towards the tumor implant in 5–6 days, eventually sweeping over the blank polymer. In test corneas, the directional growth of new capillaries from the limbal blood vessels towards the tumor occurs at a reduced rate and is often inhibited such that an avascular region around the polymer is observed (Fig. 1). This assay is quantitated by measurement of the maximum vessel lengths with a stereoscopic microscope.

In the CAM bioassay, fertilized chick embryos are placed into Petri dishes and cultured in a humidified incubator in 5% CO_2. On day 6 of development, methylcellulose (1% w/v in distilled water) discs impregnated with the test sample or an appropriate control substance are placed onto the vascular membrane at its advancing edge. On day 8, the area around the implant is observed and evaluated. Avascular zones surrounding the test implant indicate the presence of an inhibitor of embryonic neovascularization[11].

In vivo assays such as these, however, are extremely time consuming, expensive and require large amounts of precious test material. The development of techniques to culture endothelial cells[12] made possible the development of *in vitro* assays (see next section) which could function as "screens" for *in vivo* events. These assays both qualitatively and quantitatively measured the cellular and biochemical components of angiogenesis in a more rapid and reproducible manner using small amounts of test materials.

It is now established that the phenomenon of angiogenesis occurs via the "sprouting" of new capillaries from the preexisting microvasculature. Enzymatic degradation of the basement membrane of the parent microvessel is followed by capillary endothelial cell (EC) migration in response to an angiogenic stimulus. The migrating EC align themselves to form a new sprout. Trailing EC proliferate, thereby increasing the immature sprout length. Subsequently, the process of lumen formation occurs in which two hollow sprouts join to form vascular loops. Pericytes and basement membrane components surround the immature capillaries, completing the formation of the mature capillary bed[13].

IN VITRO ASSAYS FOR ANGIOGENESIS INHIBITORS

With the knowledge of the sequence of events required for neovascularization to occur and with the availability of cultured EC, the development and use of *in vitro* assays devised to expedite the discovery of angiogenesis inhibitors focused on the following components of the angiogenic process: degradation of the basement membrane, migration and proliferation of capillary EC and the formation of three dimensional capillary tubes. To the extent that a factor could inhibit one or all of these key cellular/biochemical events *in vitro*, it is then a candidate for further testing in the standard *in vivo* models.

Capillary EC proliferate in response to an angiogenic stimulus during neovascularization. Therefore, an *in vitro* assay using the cells actually involved in the process of angiogenesis was developed to mimic the process of neovascularization *in vitro*[11]. Capillary EC were stimulated to proliferate by acidic fibroblast growth factor (aFGF) a known angiogenic molecule and then challenged with varying concentrations of test substance. Following a three day incubation period, the number of endothelial cells was measured on the basis of the colorimetric measurement of acid phosphatase.[14] Results were supported by electronic cell counting. This assay is particularly useful because it provides the unique opportunity to screen large numbers of samples in a highly sensitive and reproducible manner.

A second critical event required for angiogenesis to occur is the migration of capillary EC through the extracellular matrix towards an angiogenic stimulus. Migration of endothelial cells can be tested *in vitro* using a modification of the Boyden chamber technique[15]. A blind-well Boyden chamber[16] consists of two wells (upper and lower) separated by a porous membrane. The lower wells receive a known concentration of growth factor and the upper wells receive a predetermined number of cells and inhibitor. Cells attached to the upper surface of the membrane migrate through and attach to the lower membrane surface. The membrane is then fixed and stained for counting[17].

Although a number of factors have been studied *in vitro* which can interfere with one or more of the steps required for angiogenesis, it is essential to test these substances using *in vivo* assays to ensure that angiogenesis is truly inhibited. Only those factors which have been shown to inhibit angiogenesis *in vivo* will be discussed below.

INHIBITORS OF ANGIOGENESIS

Tissue-derived inhibitors. Cartilage has been studied as a potential source of an angiogenesis inhibitor because

of its avascularity. Cartilage is a relatively tumor-resistant tissue and the tumor associated with cartilage, chondrosarcoma, is the least vascularized of all solid tumors. A number of different groups have shown that cartilage and extracts of cartilage inhibit angiogenesis *in vivo* and endothelial cell proliferation *in vitro*[10,18–22]. Partially purified extracts of cartilage have also been shown to inhibit tumor-induced neovascularization when delivered regionally (via controlled release polymer) and when delivered systemically (via infusion)[22]. The critical role that proteolytic enzymes appear to play in the process of neovascularization suggested that protease inhibitors might be anti-angiogenic agents[21–24]. Recently, an angiogenesis inhibitor from bovine scapular cartilage has been purified, characterized and amino terminal sequence data obtained[11]. This molecule, which is an acid and heat stable protein, is a collagenase inhibitor with a relative molecular mass (Mr) of 27,650.

Purified cartilage-derived inhibitor (CDI) is a powerful inhibitor of aFGF-stimulated capillary EC proliferation. Using the assay system described above for measuring capillary EC proliferation, CDI, at a concentration of 96 nM, caused 72% inhibition of proliferation. These results were supported by electronic cell counting assays and tritiated thymidine incorporation studies. Using a modification of the Boyden chamber assay, CDI inhibited capillary EC migration with an IC_{50} (the inhibitory concentration at which 50% inhibition is obtained) of 16 nM[11]. It was further tested for its ability to inhibit angiogenesis *in vivo* on the chick CAM. Purified CDI (4 μg samples) in methylcellulose discs was applied to the surfaces of growing CAMs of 6 day old fertilized chick embryos. After a 48-hour exposure of the CAMs to CDI, large avascular zones were observed as opposed to the control CAMs which never developed avascular zones (Fig. 2). CDI (4 μg = 145 pmols) is a powerful inhibitor of neovascularization *in vivo* when compared to the lowest reported doses of previously reported inhibitors discussed below.

Recently, metalloproteinases such as collagenase have been strongly implicated in the process of malignant conversion, that point in cancer progression when tumor cells gain the capacity to invade and metastasize. A correlation has been demonstrated between the acquisition of the malignant phenotype and an increase in the expression of metalloproteinases (Type IV and transin). Additionally, a key metalloproteinase inhibitor, TIMP (tissue inhibitor of metalloproteinase), is now considered to be a tumor-suppressor gene product[25]. It is interesting to note that CDI differs from TIMP isolated from human amniotic fluid (which itself is virtually identical to a human skin fibroblast inhibitor with the exception of one residue difference) in only two amino acids over the first 28 NH_2-terminal residues[11].

Other avascular tissues have also been studied as a potential sources of angiogenesis inhibitors. Vitreous extracts have been shown to inhibit neovascularization *in vivo*, the lowest reported dose being 200 μg, and endothelial cell proliferation *in vitro*[26,27]. The agent(s) responsible for this inhibition remains to be purified and it appears that vitreous may contain at least two inhibitory species, one ≤ 10 kD, the other ≤ 50 kD[26]. Extracts of human and murine lens have been shown to inhibit endothelial cell proliferation in a cell specific and reversible manner[28]. Additionally, bovine corneal extracts have recently been shown to contain an inhibitor of angiogenesis *in vivo* which appears to be a low (≤10 kD) molecular weight non-peptide inhibitor. Purification and identification of all of these factors has not yet been accomplished[29].

Angiostatic steroids. Angiogenic steroids are a unique

FIGURE 2 Inhibition of embryonic angiogenesis by CDI (cartilage-derived inhibitor) in the chick chorioallantoic membrane assay (CAM). (A) Normal CAM containing empty methylcellulose disk (B) CAM containing a methylcellulose disk impregnated with CDI surrounded by avascular zone.

group of molecules which inhibit angiogenesis. Early work on the effects of steroids on angiogenesis showed that heparin or heparin fragments with no anticoagulant activity could inhibit angiogenesis *in vivo*, using the CAM assay, at a dose of 50 μg of heparin with 60 μg of hydrocortisone, and could cause tumor tumor regression and significantly inhibit metastases in the presence of cortisone[30]. These results were important in their support of the hypothesis that an antiangiogenic therapy could ultimately influence tumor growth. Subsequent structure-activity studies with steroid analogs demonstrated that the antiangiogenic activity of the heparin-hydrocortisone combination was not a function of the mineralocorticoid, glucocorticoid or other known bioactivities of hydrocortisone and resulted in the term "angiostatic steroid"[31]. Recently, the use of a synthetic heparin substitute, β-cyclodextrin tetradecasulfate administered with angiostatic steroids, has been shown to inhibit angiogenesis at a lowest reported dose of 14 μg of β-cyclodextrin tetradecasulfate with 60 μg of hydrocortisone in the CAM assay. This drug pair has also been shown to inhibit endotoxin-induced angiogenesis in the rabbit corneal pocket assay both when delivered locally and topically[32].

Protamine. Protamine, a sperm-derived, cationic protein with a molecular weight of 4.3 kD, has been shown to be a specific inhibitor of angiogenesis, inhibiting neovascularization on the CAM at a dose of approximately 50 μg. When administered systemically, it inhibited tumor growth and metastases in a number of animal models although its efficacy is limited by its toxicity at high doses[33].

Platelet factor-4. Platelet factor-4 (PF4), a collagenase inhibitor with a very strong affinity for heparin, is a tetrameric polypeptide with a molecular weight of approximately 30 kD. It is released from platelets during aggregation as a complex with chondroitin sulfate. This protein was shown to inhibit angiogenesis in the CAM assay at a dose of approximately 10 μg[33,34]. Recent studies using

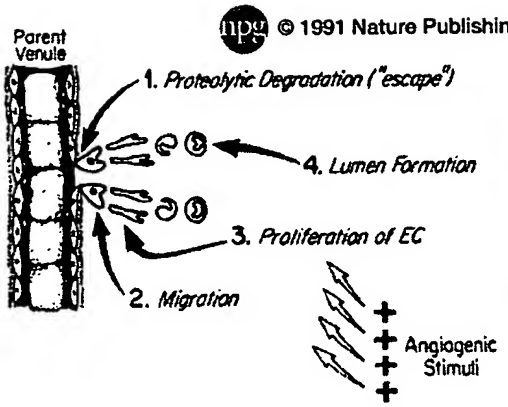

FIGURE 3 Potential intervention sites for the inhibition of neovascularization.

recombinant PF4 have further shown that it is an inhibitor of human umbilical vein endothelial cell proliferation with an IC_{50} of approximately 1 μM[34]. More recently, PF4 has been shown to inhibit the growth of murine melanoma and human colon carcinoma[35].

Thrombospondin. Recently, a 140 kD protein (Gp140) homologous in sequence and function to the C-terminus of human thrombospondin has been shown to inhibit angiogenesis *in vivo* and capillary endothelial cell migration *in vitro*[36,37]. This inhibitory factor was purified from the conditioned media of hamster and human hybrid lines when they were expressing an active cancer suppressor gene. Since the onset of angiogenesis and tumorigenesis was concomitant with the loss of GP140 activity, the production of such an angiogenic inhibitor may be a means by which tumor growth is controlled under certain circumstances[36,37]. These results are consistent with those observed for TIMP, which demonstrate that 3T3 cells which are down modulated for TIMP expression acquire the capacity for invasion and metastases[25].

Other angiogenesis inhibitors. There are a number of other angiogenesis inhibitors that can play a role in negatively modulating neovascularization. These include tumor necrosis factor-alpha (TNF-α), which has been shown to inhibit capillary and aortic cell proliferation as well as smooth muscle growth[38]. Paradoxically, TNF-α stimulates angiogenesis *in vivo*. This effect is similar to that observed for transforming growth factor beta (TGF-β) which, although it is a potent inducer of capillary tube formation *in vitro* and stimulates angiogenesis *in vivo*, is an inhibitor of aortic and capillary endothelial cell proliferation and migration *in vitro*[39,40]. Since angiogenesis can be thought of as a two stage process, one proliferative and one based on differentiation of capillary endothelial cells, it has been hypothesized that TGF-β may affect the process of neovascularization by shifting the balance towards the differentiation of capillary ECs into capillaries, accomplishing this by inhibiting their proliferation and migration[5].

Alpha interferon has been shown to inhibit endothelial cell proliferation and capillary tube formation *in vitro* and has been used as a positive therapy in patients with hemangioendotheliomas[41]. Gamma interferon is also an inhibitor of growth-factor stimulated endothelial cell proliferation as well as a number of transformed cell lines[42]. Certain antibiotics are also anti-angiogenic. Minocycline, a semisynthetic tetracycline antimicrobial with anti-collagenase properties has recently been shown to inhibit tumor-induced angiogenesis *in vivo*[43], and a family of angiogenesis inhibitors (angioinhibins), which are synthetic analogues of fumagillin, have been synthesized and shown to suppress the growth of a wide variety of solid tumors[44].

MECHANISMS OF ACTION

A potential model for the sequence of events involved in angiogenesis is one in which an angiogenic factor(s) stimulates capillary EC to produce proteolytic enzymes such as collagenase and plasminogen activator, which degrade the basement membrane of the parent venule facilitating the release of the capillary EC from the pre-existing vessel. In the case of tumor angiogenesis, it has been suggested that tumor cells might release, in addition to angiogenic factors, a chemoattractant for mast cells[45]. Mast cells might then migrate towards the tumor where they release heparin, a glycosaminoglycan, which has been shown to enhance the activity of angiogenesis factors *in vivo*[33,46] and which can potentiate growth-factor stimulated EC proliferation and migration *in vitro*[46,47]. The capillary cells, under the influence of angiogenic factors and heparin, which has also been shown to stimulate collagenase activity in EC[48], would then migrate out of the parent vessel and into the perivascular space and through the local extracellular matrix (facilitated by protease activities) towards the angiogenic stimuli. These cells, which have "sprouted" from the parent vessel, proliferate in response to angiogenic stimuli and begin to elongate the capillary sprout. Finally, these capillary EC join to form lumen and eventually, with branching, form a mature capillary network surrounded by basement membrane[13]. The inhibition of angiogenesis may be accomplished via a number of different biochemical and cellular means all focused on intervening in the process of neovascularization at these key junctures (Fig. 3).

One early event in neovascularization is the breaching of the parent venule's basement membrane to allow EC "escape" and the subsequent capillary EC migration through extracellular matrix. At this stage and throughout the process of capillary formation, EC must degrade extracellular matrix in order to move towards the angiogenic stimulus. Collagenase has been shown to be one of the key proteases required at these stages of neovascularization[23]. Additionally, collagenase has been shown to be required for tumor cells to breach the boundaries of the vascular tree during metastases[24]. The fact that the collagenase inhibitors CDI, PF4 and minocycline are anti-angiogenic is consistent with earlier work demonstrating the importance of collagenase in neovascularization and suggests that at least one way in which they exert their inhibitory effects on neovascularization might be through inhibition of this key proteolytic enzyme.

Furthermore, both CDI and PF4 ultimately cause local perturbations in the extracellular matrix, that is, they can cause changes in the integrity and/or quantity of intact basement membrane components, in this case, collagen. These changes have been shown to play a role in the control of neovascularization, since normal capillary development requires an intact basement membrane[49]. Other inhibitors such as the angiostatic steroids may work through similar mechanisms. The angiostatic steroids have been suggested to act to inhibit neovascularization by altering capillary basement membrane turnover when administered with heparin[49].

Another potential mechanism for anti-angiogenic activity focuses on the glycosaminoglycan, heparin, and its ability to enhance the effect of angiogenesis factors. For example, protamine has been shown to possess a very strong affinity for heparin as do many potent stimuli of angiogenesis (heparin-binding growth facors). It has been hypothesized that the anti-angiogenic effect of protamine is a function of its interference with these growth factors' stimulation of capillary EC functions via binding of heparin. In support of this hypothesis, it has been shown that

the mitogenic activity of soluble and matrix-bound FGF is inhibited by protamine[50]. Additionally, PF4 was originally tested for its anti-angiogenic ability due to its high affinity for heparin and it has been suggested that the mechanism by which it inhibits neovascularization may be similar to that of protamine[53].

CONCLUSION

The naturally occurring balance between endogenous stimulators and inhibitors of angiogenesis is one in which inhibitory influences predominate[37]. In those rare instances in which neovascularization occurs under normal physiological conditions, such as in wound healing, corpus luteum development and embryogenesis, it is stringently regulated and temporally and spatially delimited[37]. Under conditions of pathological angiogenesis such as that characterizing solid tumor growth, these regulatory controls fail. These observations suggest that a viable therapeutic strategy for the control of those diseases characterized by deregulated neovascularization can be developed pending the availability of potent, reliable and biologically compatible angiogenesis inhibitors.

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